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Variability in the Fatty Acid Composition of Wax Esters from Vernix Caseosa and Its Possible Relation to Sebaceous Gland Activity

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Wax ester/sterol ester (WE/SE) ratios were measured in samples of vernix caseosa lipid obtained from 21 full-term infants. The mean WE/SE ratio was twice as high in males as in females, suggesting higher average fetal sebum production rates in males, but individual values varied widely and there was considerable overlap between the sexes.

Wax esters (a class of lipid produced only by the sebaceous glands) were isolated from 6 of the 21 samples of vernix caseosa lipid and analyzed for fatty acid composition. The percentages of iso and anteiso branched saturated fatty acids and of Δ9-type monounsaturated fatty acids were found to be correlated negatively with the WE/SE ratios of the original lipid samples. Values for the percentages of the major Δ9-type monounsaturates, plotted as a function of WE/SE ratios, fell near a curve of the shape which would be expected if a limited quantity of Δ9-type monounsaturates were available for sebum synthesis, and were diluted with varying amounts of Δ6-type monounsaturates, the amount of the Δ6-type monounsaturates being proportional to the amount of wax esters synthesized by the sebaceous glands. The results suggested that the rate of sebum production may be the sole determinant of the percentage of Δ9-type monounsaturates in sebaceous wax esters and a partial determinant of the percentage of iso and anteiso branched saturates.

The activity of human sebaceous glands varies at different stages of life in response to varying levels of circulating androgens. Maximal activity is reached during puberty and maintained until menopause in women and until approximately age 70 in men [1,2]. The increase in sebaceous gland activity at puberty is accompanied by changes in the relative amounts of various lipid classes on the skin surface, especially in the amounts of wax esters relative to cholesterol and cholesterol esters [3,4]. These changes probably result mainly or entirely from an increase in the relative contribution of sebaceous lipid compared to epidermal lipid. Furthermore, there has been speculation that androgenic stimulation may alter the fatty acid composition of the lipids produced by the sebaceous glands [3,5]. Since skin surface triglycerides and free fatty acids are mixtures of sebaceous and epidermal contributions, efforts to demonstrate changes in fatty acid composition depend mainly on results obtained with wax esters, which are purely sebaceous [6,7].

Thorough analyses of the fatty acid composition of wax esters from the skin surface of an adult male and from the vernix caseosa of a male infant have been reported by Nicolaides et al [8]. These authors commented particularly on their observation that, while the unsaturated fatty acids of adult wax esters are almost entirely the products of a uniquely cutaneous Δ6-desaturation process, the unsaturated fatty acids of the vernix caseosa wax esters contained about 12% of species resulting from a Δ9-desaturation step. Nicolaides et al postulated that the Δ9-type unsaturates are synthesized in fetal sebaceous glands rather than obtained from the circulation. This conclusion apparently was based on the observation that some of these Δ9-fatty acids were not derivable, by chain extension, from oleic acid (18:1Δ9), which is the major circulating monounsaturated fatty acid, but instead consisted of palmitoleic acid (16:1Δ9) and its extension products.

Nazzaro-Porro et al [5] studied the fatty acid composition of skin surface wax esters from individuals of various ages and reported that fatty acids having the Δ9-pattern of unsaturation were higher in the wax esters of prepuberal children and the elderly than in the wax esters of individuals aged 20-65. This observation indicated that there may be a relationship between sebum production rates and the amount of Δ9-type fatty acids in wax esters.

The study of Nazzaro-Porro et al [5] included thin-layer chromatographic analyses of vernix caseosa lipid. On the basis of these analyses, they concluded that there was a clear-cut difference between the 2 sexes in the amount of sebum in vernix caseosa, with males producing much more sebum than females during fetal life. This observation suggested the present study which sought to further examine the relationship between wax ester fatty acid composition and fetal sebaceous gland activity. Since direct gravimetric measurements of fetal sebum production cannot be made, WE/SE ratios were used as a substitute measurement. Our reasons for believing that WE/SE ratios are a valid measure of fetal sebum production are outlined in the "Discussion."

We were aided in the analysis of the wax esters by a new method for separating wax esters from sterol esters using chromatography on magnesium hydroxide [9]. This adsorbent is similar in its chromatographic properties to magnesia, which was used by Nicolaides et al in their analysis of vernix caseosa lipids [8], but it has somewhat better resolving power for the separation of wax esters from sterol esters. Although silica gel has been used also as an adsorbent for isolating wax esters [3,5], in our experience skin surface wax ester and sterol ester bands invariably overlap on silica gel.

MATERIALS AND METHODS

Vernix Caseosa Lipid

Vernix caseosa was collected from the skin surface of 21 full-term, healthy infants using gauze sponges or tongue depressors. Samples which appeared to be excessively contaminated with blood were discarded. When the lipid extracts were analyzed chromatographically (see next paragraph) some were found to be contaminated with a nonpolar, nonlipid material, whose source we could not identify. K-Y jelly was checked and exonerated. The material had a lower chromatographic mobility than the lipid classes in which we were interested and did not interfere with our analyses.

WE/SE Ratio

100 μg samples of vernix caseosa lipid were applied to 6 mm lanes scored in 0.25-mm thick layers of silica gel G spread on 20 x 20 cm thin-

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Abbreviations:
HPLC: high pressure liquid chromatography
TLC: thin layer chromatography
WE/SE: wax esters/sterol esters

layer chromatography (TLC) plates. The plates were developed to the top with hexane and then with toluene. The developed chromatograms were charred by spraying with 50% aqueous sulfuric acid and then heating slowly to 220° on a hot plate covered with a 20 x 20 cm aluminum slab. When they had cooled, the charred chromatograms were quantified [10] using a photodensitometer (Clifford 445, Corning Medical Products, Medfield, MA). The overlapping wax ester and sterol ester peaks were triangulated manually, a correction factor was applied to compensate for the nonlinearity of photodensitometer response [11], and the WE/SE ratio was calculated.

Isolation of Wax Esters

A wax ester + sterol ester (monoester) fraction was separated from other vernix caseosa lipid classes by preparative TLC on 1 mm thick layers of silica gel G developed as for the analytical plates described above. Bands were detected by spraying the plates with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol and subsequent viewing of the plates under ultraviolet light. The area of silica gel containing the monoesters was scraped from the plate and the lipid was eluted from the adsorbent with ether.

Wax esters were separated from sterol esters by chromatography on magnesium hydroxide [9]. A 120 x 2 cm high pressure liquid chromatography (HPLC) column packed with magnesium hydroxide was used in conjunction with 2 HPLC pumps connected in parallel, an injector, and a refractive index detector (all HPLC equipment from Waters Associates, Milford, MA). A 10–50 mg sample of monoesters was injected into the system and eluted from the column with 2% ethyl acetate in hexane flowing at 19.8 ml/min. The complete separation required about 40 min.

Fatty Acid Composition of Wax Esters

The wax esters were saponified by heating to 70° for 2 hr with 1N KOH in 20:1 methanol-water and the liberated fatty acids were methylated by adding an excess of BCl_3-methanol directly to the saponification mixture. Methyl esters were isolated by TLC on 1-mm thick layers of silica gel G developed with toluene. The methyl esters were then separated by degree of unsaturation on 0.5 mm layers of silica gel H-10% $AgNO_3$ developed twice in toluene. The amounts of saturated, monounsaturated, and diunsaturated methyl esters recovered were estimated by TLC on silica gel G of aliquots of each fraction. Known amounts of methyl oleate were run on the same plate for comparison of photodensitometric peak areas. The methyl ester fractions from argentation TLC were analyzed on a 4 mm x 6 ft column packed with 10% DEGS installed in an F&M 402 gas chromatograph (Hewlett Packard Co.). Peaks were integrated with an electronic integrator (Columbia Scientific Industries, Austin, TX, model 204). Structures were identified by plots of log (retention time) versus probable number of carbons and by reference to Nicolaides's elucidation of the structures of the branched fatty acids of vernix caseosa [12].

To measure the relative amounts of the various double-bond positional isomers in the monounsaturates, 1-5 mg samples of monounsaturates were first fractionated by chain length on a 20 ft x 7 mm copper column packed with 10% SE-30 using a Varian model 3760 gas chromatograph. A nondestructive, thermal conductivity detector was used. A heated tube carried the effluent from the detector to a fraction collector (Packard Instruments, Downers Grove IL, model 850). Emerging peaks were trapped in cartridges packed with celite and later eluted with ether. Straight and branched methyl esters of the same molecular weight were collected together. Fractions of uniform molecular weight were oxidatively cleaved and the fragments analyzed by the pyrolysis methylation method of Downing and Greene [13], except that a 0.02 in x 150 ft stainless steel column, wall-coated with Silar 10 C (Perkin-Elmer Corp., Norwalk, CT), was used instead of the packed column originally described. Straight and branched monocarboxylic fragments were distinguished easily.

TABLE I. *Influence of infant's sex on the composition of vernix caseosa lipid*

Sex	n[b]	WE/SE[a]	
		Mean ± SD[c]	Range
Male	14	0.71 ± 0.53	0.18–1.84
Female	7	0.34 ± 0.25	0.14–0.71

[a] The ratio (by weight) of wax esters to sterol esters was measured by quantitative TLC.
[b] n = number of newborns studied.
[c] SD = standard deviation. Student's *t*-test indicated a significant difference between the means ($p < 0.05$).

TABLE II. *Composition of the major saturated fatty acids of 6 samples of vernix caseosa wax esters*[a]

Carbon skeleton[b]	% of saturates					
	F(0.17)[c]	F(0.20)[c]	M(0.47)[c]	M(0.67)[c]	F(0.69)[c]	M(1.84)[c]
12 st	—	—	0.5	1.7	1.0	2.3
13 Mebr	—	—	0.2	1.1	1.0	2.1
13 ai	—	2.1	0.2	0.6	—	0.3
13 st	—	—	—	0.6	—	0.9
14 i	2.3	14.5	17.7	17.3	16.2	9.6
14 st	0.9	3.2	7.8	10.0	10.6	13.8
15 Mebr	—	—	—	1.5	1.1	4.1
15 ai	14.1	31.8	19.5	19.9	13.0	12.9
15 st	1.2	1.6	2.4	3.1	3.1	4.8
16 Mebr	—	0.9	—	0.4	2.9	2.7
16 i	25.0	17.3	20.4	13.2	13.2	7.5
16 st	15.2	8.0	13.8	14.6	19.7	21.6
17 Mebr	—	—	1.7	1.7	2.4	3.3
17 ai	22.4	14.5	8.4	7.8	6.5	4.1
17 st	0.9	0.7	1.3	1.0	1.2	1.4
18 i	5.1	2.1	2.3	1.4	1.2	0.6
18 st	8.9	2.6	2.2	2.9	6.7	6.5
19 ai	1.2	0.5	0.4	0.4	—	0.2
20 i	3.0	0.5	1.2	1.0	tr	0.7
20 st	—	—	—	—	tr	0.5
Straight						
Even	25.0	13.8	24.3	27.5	37.0	42.4
Odd	2.1	2.3	3.7	4.7	4.3	7.1
Branched						
Iso	35.4	34.4	41.6	32.9	30.6	18.4
Anteiso	37.7	48.9	28.5	28.7	19.5	17.5
Mebr	0	0.9	1.9	4.7	7.4	12.2

[a] —, not detected; tr, trace.
[b] st, straight; Mebr, monomethyl branching other than iso (i) or anteiso (ai).
[c] F, female; M, male; WE/SE ratio in ( ); M(0.47) is a pooled sample from three male infants with identical WE/SE ratios.

TABLE III. *Composition of the major monounsaturated fatty acids of 6 samples of vernix caseosa wax esters*[a]

Carbon skeleton	Double bond position	(% of monoenes) % of skeletal type					
		F(0.17)[b]	F(0.20)[b]	M(0.47)[b]	M(0.67)[b]	F(0.69)[b]	M(1.84)[b]
Straight							
14		(0.9)	(3.0)	(4.2)	(7.7)	(6.7)	(9.9)
	Δ6	NA	NA	NA	100	100	100
15		(2.5)	(2.8)	(2.9)	(4.4)	(5.1)	(5.7)
	Δ5	NA	NA	NA	8	—	—
	Δ6				92	100	100
16		(25.1)	(27.7)	(38.1)	(37.1)	(43.8)	(46.6)
	Δ6	80	64	89	96	94	96
	Δ7	—	—	2	—	3	—
	Δ8	—	—	1	—	2	2
	Δ9	20	36	8	4	4	2
17		(1.6)	(3.2)	(2.0)	(2.2)	(1.8)	(3.1)
	Δ6	NA	46	62	74	60	71
	Δ7		—	—	—	—	10
	Δ8		17	20	15	23	10
	Δ9		37	18	11	16	9
18		(40.3)	(33.0)	(14.6)	(11.8)	(12.9)	(7.9)
	Δ6	6	3	7	8	5	19
	Δ7	—	2	2	—	5	5
	Δ8	14	11	17	23	31	42
	Δ9	49	51	40	39	40	24
	Δ10	8	4	2	4	5	2
	Δ11	23	29	33	21	14	9
Branched[c]							
15ai		(0.4)	(1.0)	(0.9)	(0.8)	(1.3)	(0.9)
	Δ6	NA	NA	NA	100	100	100
16i		(19.2)	(14.9)	(29.2)	(26.4)	(23.0)	(18.4)
	Δ6	100	100	100	100	100	100
17ai		(5.8)	(10.9)	(5.1)	(6.8)	(3.4)	(6.0)
	Δ6	NA	NA	100	NA	NA	NA
18i		(4.2)	(2.8)	(3.0)	(2.4)	(2.1)	(1.4)
	Δ6	33	NA	58	59	NA	NA
	Δ8	67		42	41		
Straight		70.4	69.7	61.8	63.2	70.3	73.2
Branched		29.6	29.6	38.2	36.4	29.8	33.2

[a] NA, not analyzed; —, not detected.
[b] F, female; M, male; wax ester/sterol ester ratio in ( ); M(0.47) is a pooled sample from 3 male infants with identical WE/SE ratios.
[c] i, iso; ai, anteiso.

RESULTS

WE/SE Ratios

Vernix caseosa lipids from 21 infants were examined by quantitative TLC. The results (Table I) indicated that there was a significant difference between boys and girls in mean WE/SE ratios, but that there was also wide overlap between the sexes in this parameter.

Wax Ester Fatty Acid Composition in Relation to WE/SE Ratios

Of the 21 vernix caseosa samples collected, specimens from 2 male and 3 female infants contained sufficient lipid for a complete analysis of wax ester fatty acid composition. Another specimen was prepared by combining samples from 3 male infants who had identical WE/SE ratios but insufficient lipid for separate analysis. WE/SE ratios for the 6 samples covered a 10-fold range from 0.17 to 1.84. Pure wax esters were isolated from these 6 samples and the constituent fatty acids were recovered as the methyl esters in preparation for analysis.

Because of the complexity of wax ester fatty acid composition, it was necessary to separate the methyl esters derived from wax esters by degree of unsaturation before gas chromatographic analyses could be done. For the 6 samples, the average distribution of the wax ester fatty acids by degree of unsaturation was 30% saturates, 66% monounsaturates, and 4% diunsaturates, as estimated by quantitative TLC after recovery from preparative argentation TLC. There was some variation between the individual samples in the percentages (e.g., saturates varied from 18-41%) but this variability may have been largely experimental, since losses of material can result from preparative TLC. Because of the possibility of losses, the results in Tables II and III are presented as percentages within each class for saturates and monounsaturates. Diunsaturates were not analyzed.

The composition of the saturated fatty acids from vernix caseosa wax esters (Table II and Fig 1) varied among individuals, most noticeably in the relative amounts of branched chains. The relationship between the relative amounts of iso and anteiso type fatty acids and the WE/SE ratio is shown more clearly in Fig 1. It can be seen that the proportion of iso or anteiso in each chain length tends to be less in samples with higher WE/SE ratios. In contrast, there was a positive correlation between the proportion of fatty acids with monomethyl branching other than iso or anteiso and the WE/SE ratio (Table II).

The composition of the monounsaturated fatty acids in vernix caseosa wax esters, including the double bond positions for those chain lengths for which sufficient material was available, is shown in Table III. In contrast to the results with the saturates, most of the fatty acids have straight chains and the relative amounts of straight and branched acids do not vary with the WE/SE ratio. However, the relative amounts of various species of straight-chain acids are variable. It can be seen that the percentage of 18-carbon straight chains correlates negatively with the WE/SE ratio, and there is a corresponding increase in the percentages of 14- and 16-carbon straight chains (Table III, the columns of numbers in parentheses showing the percent of monoenes represented by each skeletal type, irrespective of double bond positions). When the percentages of

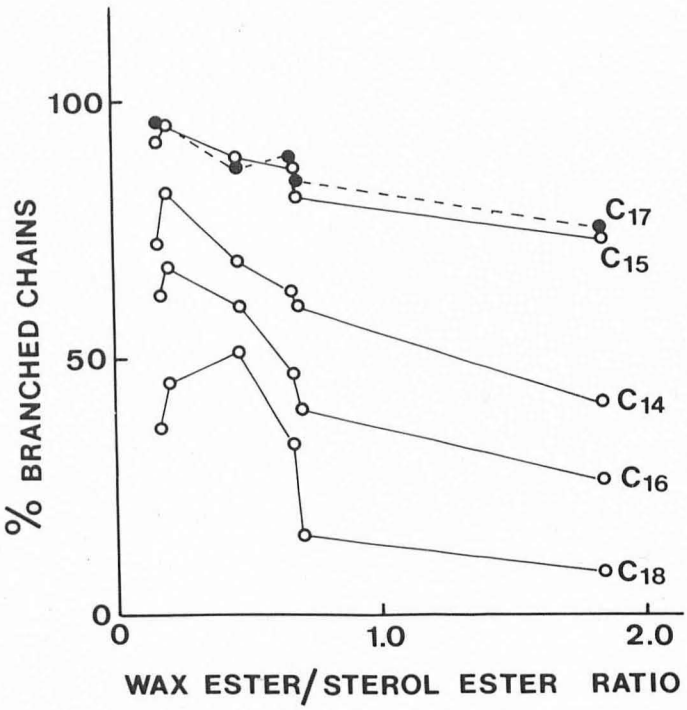

FIG 1. The relationship between the WE/SE ratio of vernix caseosa lipid and the percentage of iso or anteiso branched isomers in the 14-, 15-, 16-, 17- and 18-carbon saturated fatty acids from vernix caseosa wax esters. Branched fatty acids with even numbers of carbons are iso, and branched fatty acids with odd numbers of carbons are anteiso.

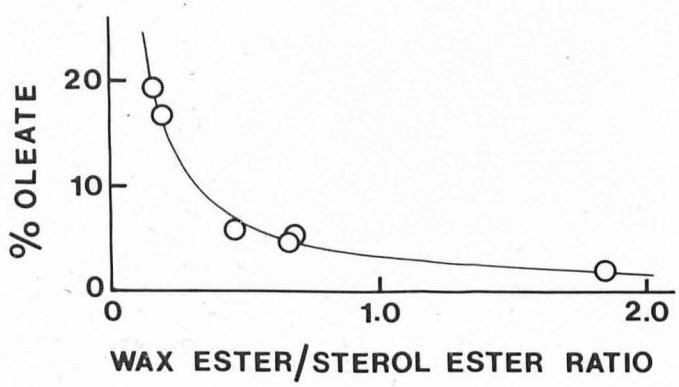

FIG 2. The relationship between the WE/SE ratio of vernix caseosa lipid and the % oleate in the monoenoic fatty acids of vernix caseosa wax esters. The points fall near the curve (solid line) of the equation % oleate = 3.25/(WE/SE).

the various double bond isomers in each skeletal type are considered (Table III, columns without parentheses), it can be seen that there were higher concentrations of oleic acid, palmitoleic acid, and the 18:1Δ11 acid in samples with low WE/SE ratios.

The results of Table III suggested that the percentages of oleic acid and of monounsaturates of the palmitoleic type in wax esters may be controlled by a dilution mechanism. In Fig 2 the percentage of oleic acid in wax ester monoenoic fatty acids (% of 18:1 in monoenes × % of 18:1Δ9 in 18:1) was plotted as a function of the WE/SE ratio. The data fit closely the empirical equation % oleate = 3.25/(WE/SE). This relationship implies that for every doubling in the WE/SE ratio, the % oleate will be halved. In agreement with Nicolaides et al [8], the percentage of palmitoleate plus its extension product, 18:1Δ11, approximately equalled the percentage of oleate. This was the case for every sample, so the sum of these two fatty acids followed the

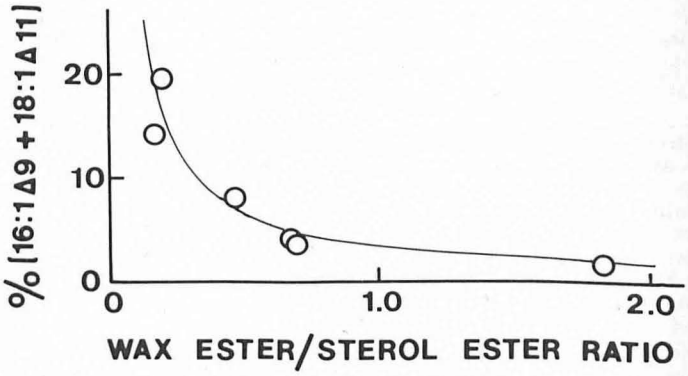

FIG 3. The relationship between the WE/SE ratio of vernix caseosa lipid and the %(16:1Δ9 + 18:1Δ11) in the monoenoic fatty acids of vernix caseosa wax esters. The equation represented by the solid line is the same as in Fig 2.

same dilution curve as oleate (Fig 3). From the results of Nicolaides et al [8], the coordinates of a point for Fig 2 can be calculated as % oleate, 4.2 and WE/SE ratio, 0.63. This point falls fairly close to the curve for our data. For Fig. 3, their point would be %(16:1Δ9 + 18:1Δ11), 5.5 and WE/SE ratio 0.63, which is very close to our curve.

DISCUSSION

The aim of this study was to determine whether the fatty acid composition of a sebaceous lipid, i.e. wax esters, varied with the rate of sebum production. Vernix caseosa presented an advantage for this study in that the relatively large amounts of skin surface lipid which were necessary for the analysis of wax esters were easily obtained. However, a disadvantage of this source was that sebum production rates could not be measured directly. It seemed reasonable to assume, however, that the WE/SE ratios of vernix caseosa lipid would be a measure of the accumulation of fetal sebum relative to the lipid derived from keratinizing epidermis. Evidence linking sebum excretion rates with concentrations of sterol esters in skin surface lipids comes from studies of anatomical variations in skin surface lipid composition and from studies of children. Thus, for anatomical areas with low rates of sebum secretion, a negative correlation between sebum excretion rate and the concentration of cholesterol esters in skin surface lipids has been reported [7]. Likewise, during childhood, when sebum excretion rates are low, cholesterol ester concentrations are high, and decrease when sebum excretion rates increase at puberty [4].

On the other hand, Nicolaides has argued, on the basis of the fatty acid composition of vernix caseosa sterol esters, that both the sebaceous glands and the epidermis are major sources of the sterol esters in vernix caseosa [8]. Even if this is the case, however, it is possible that the relative amount of sterol esters produced by the sebaceous glands would vary with the activity of the glands, with the more active glands tending toward the adult pattern of producing little sterol ester compared to wax ester [7]. Moreover, the results represented in Fig 2 and 3, which indicate a good correlation between WE/SE ratios and the percentages of Δ9-type monounsaturated fatty acids in wax esters, suggest that a common factor, most likely the rate of sebum production, influences both WE/SE ratios and wax ester composition.

The higher average WE/SE ratios in vernix caseosa lipid from male infants may be the result of stimulation of the fetal sebaceous glands by testosterone. During the period when masculine differentiation of the urogenital tract is occurring (weeks 7 to 18 approximately) the fetal testis is active [14] and fetal blood testosterone levels are nearly as high as those of adult males [15]. We were surprised, however, at the extent of overlap between males and females, in view of the report of

Nazzaro-Porro et al that vernix caseosa lipid composition is diagnostic for the sex of the infant [5]. On the other hand, our results are analogous to the situation in adults, where rates of sebum production (measured gravimetrically) vary widely among individuals and there is overlap between the sexes, but where group means between males and females are significantly different [2].

Our results suggest a relationship between sebum production rates and the relative amounts of straight and branched chain saturated fatty acids in sebaceous lipids. Judging by our results, active glands excrete lipid with a lower proportion of fatty acids with terminal branching (iso and anteiso) and a higher proportion of straight chain acids and of acids with interior branching. The terminal portions of iso and anteiso fatty acids are thought to be derived from exogenous precursors. Possibly these precursors become less available in the interior of larger, more active glands, and those fatty acids which can be synthesized entirely *de novo* by the glands tend to be made preferentially.

As mentioned in the "Results" section, the shape of the curve relating WE/SE ratios to the percentages of various Δ9-type monounsaturates suggest that a dilution mechanism is operating. The following model could perhaps account for a dilution effect. Only undifferentiated sebaceous cells in the germinative layer of a sebaceous acinus may contain a Δ9-desaturase acting on palmitic acid. Since these cells are in contact with the circulation, they could also take up circulating oleic acid. After they start to differentiate and to move towards the interior of the gland, their characteristically sebaceous Δ6-desaturase becomes predominant. Moreover, in the unvascularized interior of the sebaceous acinus, circulating fatty acids may no longer be available. Therefore, the Δ9-type fatty acids would become progressively diluted by Δ6-type fatty acids synthesized *de novo* in the sebaceous cells. The relative amounts of odd- to even-numbered double bond positions in the excreted lipid would depend on the amount of fatty acid synthesized by the cell during its differentiated stage as compared to the amount incorporated into lipid during its undifferentiated stage. Cells of more active glands may synthesize more lipid during differentiation and in so doing dilute the lipids containing Δ9-type fatty acids to a greater extent.

This model could also account for the excretion of higher concentrations of sterol esters by fetal sebaceous glands, if, in fact, this occurs. In the sebum of adults, squalene accounts for about 12% of the total lipid [7]. This observation has led to the belief that sebaceous glands convert squalene to cholesterol only inefficiently, if at all. However, the germinative cells at the periphery of the glands may be metabolically different or they may take up large amounts of cholesterol from the circulation for conversion to sterol esters. As the cells differentiate, their store of sterol esters would be diluted by sebaceous squalene, wax esters, and triglycerides. In the relatively inactive glands of the fetus, this dilution effect would be less than in the active glands of an adult. This mechanism, which would link the dilution of the sterol esters to that of the Δ9-type unsaturates, could perhaps explain the close correlation between WE/SE ratios and percentages of Δ9-type unsaturates in wax esters.

This model suggests that the cells of active sebaceous glands should be larger than those of less active glands, since they would synthesize and store more lipid. Tosti [16] estimated that the cells of the large sebaceous clusters (sebaceous follicles) on the face, where sebum production is high, are larger than those of other adult glands. However, Ebling [17] has shown that in the rat, testosterone, in stimulating sebum production, also stimulates the rate of division of sebaceous cells. This result implies that more cells, rather than larger ones, are the basis of increased sebum production in the rat, although Ebling did not actually measure cell size. On the other hand, when Ebling administered estrogen to castrated rats, a reduction in sebum production occurred without an accompanying decrease in the rate of cell division [17]. It appears, therefore, that changes in the amount of lipid synthesis per cell may occur in certain circumstances. Any such changes in lipid synthesis would presumably be accompanied by changes in cell size.

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